EXHIBIT 21

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Clinical Applications of Gene Therapy for Cancer

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The number of gene therapy protocols for the treatment of cancer is growing rapidly. The most common type of approved clinical trial for cancer gene therapy involves the ex vivo gene transfer of cytokine genes (e.g., tumor necrosis factor, interleukin-2, granulocyte-macrophage colony-stimulating factor) into tumor cells. The idea behind this approach is to use gene transfer to induce a patient's tumor to become more immunogenic. The genetically altered tumor cells are reinjected into the patient in an effort to induce a systemic antitumor immune response against residual tumor cells. In other trials, investigators are using in situ gene transfer to selectively destroy cancer cells, sparing normal tissues. Continuing advances in molecular biology are likely to allow the development of new cancer treatments and methods of cancer prevention that will redefine cancer therapy.

Indexing Terms: *clinical trials/bystander effect*

Significant advances in human genetics have occurred since the structure of DNA was identified by Watson and Crick 40 years ago. By 1993, >3000 of the >100 000 genes in the human genome had been mapped. This growth in our understanding of the fundamentals of human genetics led to the initiation of the first human gene therapy experiment (*1*). On September 14, 1990, at the National Institutes of Health, a 4-year-old girl with severe combined immunodeficiency due to adenosine deaminase deficiency received the first gene therapy. This child has manifested significant improvement in her immune system as a result of the gene therapy. Over the past 3 years, the number of gene therapy trials has increased to >50 and includes several for the treatment of brain tumors. Table 1 lists the approved trials for the treatment of cancer.

Current Gene Therapy Clinical Trials for Brain Tumors

Gene therapy was applied to the treatment of brain tumors in December 1992 (*2*). In this protocol, murine fibroblast cells producing retroviral vectors (VPC)[1] were directly implanted into growing brain tumors in human patients. The gene that was transferred into the surrounding brain tumor cells was the herpes simplex–thymidine kinase (HS-tk) gene, which confers a sensitivity to the anti-herpes drug ganciclovir (Cytovene or GCV), resulting in cell death (*3, 4*). In a series of animal experiments, this technique resulted in gene transfer into an average of 60% of tumor cells and was capable of mediating complete tumor destruction in mice with experimental tumors (*5*). This destruction occurred despite the fact that <100% of the tumor cells contained the HS-tk gene. Studies in mice suggest that if at least 10% of the tumor cells in a mixture contain the HS-tk gene, >50% of the cancers can be completely eliminated. This phenomenon of destruction of adjacent non-HS-tk-containing tumor cells is called the "bystander" effect. The mechanism of the bystander effect is not completely understood. The leading hypothesis is that the phosphorylated forms of GCV pass via gap junctions into

Table 1. Approved human gene therapy experiments for cancer in the US.

Type of Cancer	Tissue	Gene
Brain tumors	Tumor cells	HS-tk
Brain tumors	Tumor cells	Antisense IGF-1
Brain tumors	HSC	MDR-1
Breast cancer	Fibroblasts	IL-4
Breast cancer	HSC	MDR-1
Colorectal cancer	Tumor cells	IL-2 or TNF
Colorectal cancer	Fibroblasts	IL-2 or IL-4
Colorectal cancer	Tumor cells	HLA-B7+β2m[a]
Malignant melanoma	T-cells	TNF
Malignant melanoma	Tumor cells	TNF or IL-2
Malignant melanoma	Fibroblasts	IL-4
Malignant melanoma	Tumor cells	γ-Interferon[a]
Malignant melanoma	Tumor cells	B7 costimulatory molecule
Malignant melanoma	Tumor cells	HLA-B7 or HLA-B7+β2m[a]
Neuroblastoma	Tumor cells	IL-2
Non-small-cell lung cancer	Tumor cells	Antisense K-ras or WTp53[a]
Ovarian cancer	HSC	MDR-1
Ovarian cancer	Tumor cells	HS-tk
Renal cell carcinoma	Tumor cells	IL-2, TNF, or GM-CSF
Renal cell carcinoma	Fibroblasts	IL-4
Small-cell lung cancer	Tumor cells	IL-2
Solid tumors	Tumor cells	HLA-B7+β2m[a]

[a] In vivo gene transfer protocols.

HSC, hematopoietic stem cells; TNF, tumor necrosis factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; β2m, β_2-microglobulin.

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[1] Nonstandard abbreviations: VPC, retroviral vector producer cells; HS-tk, herpes simplex–thymidine kinase; GCV, ganciclovir; IGF-1, insulin-like growth factor-1; MDR-1, multiple drug resistance type 1; and TIL, tumor-infiltrating lymphocytes.

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neighboring tumor cells (*6, 7*). No associated toxicity or evidence of systemic spread of the retroviral vectors has been seen with this form of in vivo gene transfer.

Eight patients with recurrent glioblastoma multiforme or metastatic tumors have been treated with the stereotactic implantation of HS-tk VPC without any evidence of toxicity related to the implantation or GCV treatment. Five have demonstrated evidence of antitumor efficacy with a decrease in size and cystic changes within the tumor. Three additional clinical trials have been approved with this technique. The first is a trial in adult patients that will focus on the direct injection of HS-tk VPC into the walls of the tumor bed at the time of tumor resection. Repeated injections of VPC will be administered through an Ommaya reservoir into the tumor bed. Two additional trials will apply this technique to recurrent astrocytomas in children.

Another approach to the treatment of brain tumors targets one of the methods that tumors appear to use to hide from the immune system. This method uses an antisense copy of insulin-like growth factor-1 (IGF-1) to block tumor cell production of IGF-1 (*8*). Insertion of an antisense IGF-1 gene into IGF-1-producing tumor cells inhibits IGF-1 production. The injection of these genetically altered cells into animals results in immunologic rejection of the cells as well as of nongenetically altered tumor cells at other sites in the body. A human clinical trial is expected to begin in early 1994.

Theoretically, the genetic manipulation of hematopoietic stem cells may also be used to protect these cells from the toxic effects of chemotherapy. This approach to the gene therapy of cancer may be accomplished by the insertion of the multiple drug resistance type 1 (MDR-1) gene into hematopoietic stem cells before administration of high-dose, myelosuppressive chemotherapy (*9*). The MDR-1 gene was isolated from tumor cells and functions to pump chemotherapy drugs (i.e., daunorubicin, doxorubicin, vincristine, vinblastine, VP-16, VM-26, taxol, actinomycin-D) out of the tumor cells. This tumor resistance mechanism is one of the ways in which tumors develop resistance to chemotherapy drugs. The use of ex vivo retroviral vector-mediated insertion of the MDR-1 gene into murine marrow cells has demonstrated significant protective effects in vivo in animals that were treated with high doses of taxol. Human clinical trials are being planned to study these properties in the high-dose chemotherapy treatment of brain tumors and disseminated breast and ovarian cancer.

Gene Therapy Trials for Non-CNS Malignancies

Major advances in our understanding of the genetic basis of cancer allow entirely new approaches to the treatment of cancer (Table 2). For instance, the deletion of tumor suppressor genes can, theoretically, be corrected at the genetic level by the insertion of a normal copy of the gene, perhaps before the development of cancer. Likewise, the overexpression of an oncogene may be blocked at the genetic level by the insertion of an antisense gene that will block oncogene expression.

A study in which the supernatant fluid from retroviral vectors will be injected directly into endobronchial lung cancers has been approved. In this experiment, the retroviral vectors will carry genes that target the genetic mechanisms responsible for the malignancy. If lung tumors are deficient in the *p53* tumor suppressor gene, a *p53* vector will be used to transfer a normal copy of the *p53* gene (*10*). In lung cancers that overexpress the K-*ras* oncogene, a vector containing an antisense K-*ras* gene will be used (*11*). The antisense K-*ras* vector will produce mirror-image RNA molecules that will bind the molecules being produced by the oncogene. These RNA:RNA hybrids will then be degraded by the cell. Experiments in animals show that inserting either the tumor suppressor gene or the antisense oncogene can result in destruction of the injected tumor in vivo.

Table 2. Potential applications of gene therapy for treatment of cancer.

1. Enhance the immunogenicity of the tumor by inserting genes for: cytokines; allogeneic surface antigens; costimulatory molecules; inhibition of tumor growth factor production with antisense IGF-1.
2. Genetically alter immune cells to increase antitumor efficacy by the insertion of: immune-stimulatory factors (e.g., IL-2) or genes that encode antibody to tumor-specific receptors to enhance tumor:immune cell interactions.
3. Insert a "sensitivity" or "suicide" gene into the tumor: HS-tk/GCV; cytosine deaminase/5-fluorocytidine; genes that disrupt DNA repair (e.g., antisense DNA polymerase).
4. Block oncogene expression: antisense oligonucleotides; ribozymes.
5. Insert tumor suppressor genes.
6. Genetically protect tissues from the systemic toxicities of chemotherapy: insertion of a MDR-1 gene.

The most common clinical trials of human gene therapy involve the injection of gene-modified human autologous or allogeneic tumor cells. These initial experiments are an attempt to immunize tumor-bearing patients against their own tumor by injecting genetically altered cells that stimulate host antitumor immunity. Human trials have been approved for in vitro insertion of the genes for interleukin-2 (IL-2), tumor necrosis factor, granulocyte–macrophage colony-stimulating factor, or B7 costimulatory molecules, by means of retroviral vectors, into melanoma, neuroblastoma, colorectal, and renal cell carcinoma cells (*12–15*). These "tumor vaccines" are produced after surgical resection of the tumor. Tumor cells are genetically altered in the laboratory by murine retroviral vectors, and once the cells have been shown to produce the inserted gene product, they are reinjected subcutaneously into the patient. Because tumor cells cannot always be grown from patients, two groups of investigators are mixing genetically modified (i.e., by IL-2 or IL-4) autologous fibroblasts with irradiated autologous tumor cells in an effort immunize patients with colorectal cancer, melanoma, breast cancer, and renal cell carcinoma. Melanoma, renal cell carcinoma, and colorectal cancer have been the primary focus of the tumor vaccine studies because they may be more immunogenic than other tumors and therefore more likely to respond to this approach. An-

other group plans to create cytokine-producing cells in situ by the direct injection of retroviral vector supernate containing an interferon-γ vector into melanoma tumor deposits. At this time, neither of these research groups has begun human experimentation.

The first trials of non-viral-mediated genetic modification of tumors in situ were initiated in 1992. For this protocol, liposomes injected directly into the tumor are used to deliver a foreign HLA-B7 gene in an attempt to increase the immunogenicity of malignant melanoma (*16*). The liposomes are taken up by tumor cells by phagocytosis. The tumor cells express the foreign HLA-B7 antigen transiently on their surface. Animal studies have shown that tumor cells expressing foreign histocompatibility antigens on their cell surface induce a significant antitumor immune response (*17*). In preliminary observations on three patients in the initial human clinical trial, one has responded, and there is no evidence of toxicity in any of the patients (*18*). In the one responding patient, not only did the injected lesion regress, but other nontreated lesions responded as well. The protocol has now been amended to include the β_2-microglobulin gene in addition to the HLA-B7 gene in an effort to further enhance immune reactivity to the tumor cells. Applications of this gene transfer method have been expanded to include solid tumors and colorectal cancer metastases to the liver.

Another approach to the genetic therapy of cancer is gene transfer into T-lymphocytes. Because T-lymphocytes are critical for the prevention and elimination of tumors, T-lymphocytes have been grown from tumor biopsies. The gene for tumor necrosis factor-α has been inserted into these tumor-infiltrating lymphocytes (TIL) in an effort to increase their antitumor efficacy (*19*). These human experiments, currently in a phase I trial, have been slowed because of poor efficiency of gene transfer into human TIL and down-regulation of cytokine expression by the TIL (*20*).

Several months ago, a human clinical trial for ovarian cancer was initiated, involving the intraperitoneal injection of irradiated human allogeneic ovarian tumor cells transduced with the HS-tk gene. It is hoped that the administration of GCV to these patients will result in destruction of the HS-tk-positive cells and produce a bystander tumor cell factor that will destroy adjacent HS-tk-negative tumor cells (*6, 7*).

The Future

As the enthusiasm for the potential use of gene therapy grows, significant hurdles remain. First, the identification of genes is occurring at a much faster rate than the development of optimal in vivo gene delivery methods. Second, as new genes are discovered, there is still a significant period of time required to develop an understanding of gene expression and regulation. Advances in these areas are required to allow for the widespread application of gene therapy. As these discoveries occur, the first patients to benefit will most likely be those with cancer. Over the next 5–10 years, gene therapy will become a standard therapy for certain forms of cancer. With the development of efficient gene transfer methods, the dream of genetic therapy for congenital and acquired diseases will become a reality.

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